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Phosphorylation of connexin-43 at serine 262 promotes a cardiac injury-resistant state

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Protein kinase Cϵ

Aims The cardioprotective agent fibroblast growth factor 2 (FGF-2) was found previously to promote phosphorylation of connexin-43 (Cx43) at protein kinase C (PKC) sites such as serine (S) 262 at levels above those of non-stimulated hearts. We asked if other PKC-dependent cardioprotective treatments cause a similar effect, and if Cx43 phosphorylation at S262 mediates resistance to injury.
Methods and results Isolated perfused adult rat hearts were subjected to the following treatments: ischaemic preconditioning (PC); diazoxide perfusion; FGF-2 pre-treatment followed by 30 min global ischaemia; 30 min global ischaemia followed by 60 min reperfusion in the presence or absence of FGF-2. Cx43 phosphorylation was assessed by western blotting with phospho-specific antibodies. Neonatal cardiomyocyte cultures were used to examine the effect of expressing Cx43 incapable of being phosphorylated at S262 due to an S to alanine (A) substitution on simulated ischaemia-induced cell death (TUNEL staining) and injury (lactic dehydrogenase release). Ischaemic PC, diazoxide, and FGF-2 pre-ischaemic or post-ischaemic treatments elicited a P*Cx43 state, defined as above-physiological levels of phospho-S262-Cx43 and phospho-S368-Cx43. P*Cx43 was sustained during global ischaemia and was accompanied by attenuation of ischaemia-induced Cx43 dephosphorylation and prevention of Cx43 lateralization. Post-ischaemic FGF-2 treatment also diminished dephosphorylated Cx43. Modest overexpression of S262A-Cx43, but not wild-type Cx43, exacerbated cardiomyocyte death and injury caused by simulated ischaemia *in vitro*. It also prevented the cytoprotective effects of FGF-2 or over-expressed PKCϵ.
Conclusions P*Cx43 marks a state of enhanced resistance to ischaemic injury promoted by PKC-activating treatments such as FGF-2 administration or ischaemic PC. Cx43 phosphorylation at S262 likely mediates PKCϵ-dependent cardioprotection.

1. Introduction

Connexin-43 (Cx43) is an integral membrane phosphoprotein, and the main constituent of cardiac gap junctions (GJ). GJ maintain electrical and metabolic coupling between cardiomyocytes and are essential for coordinated myocardial function.[1] Abnormalities in cardiac GJ promote cardiac arrhythmias, a major complicating feature of many cardiac pathologies.[2] Ischaemia-associated conduction abnormalities have been linked to Cx43 changes, such as dephosphorylation and redistribution away from intercalated disks (lateralization), followed by Cx43 degradation.[3] Prevention or reversal of pathology-associated Cx43 changes would be expected to enhance any therapeutic strategy against heart injury and disease. One such experimental strategy, ischaemic preconditioning (PC), prevents ischaemia-induced Cx43 dephosphorylation, and this is expected to contribute to protection.[4]

Fully dephosphorylated (D-) Cx43 is recognized by specific antibodies (#13-8300) and has an electrophoretic mobility of 41 kDa.[3,5] As Cx43 becomes phosphorylated at increasing number of sites, it exhibits correspondingly slower electrophoretic mobility. In the normal heart, Cx43 is phosphorylated at multiple sites resulting in electrophoretic migration at 45 kDa.[6] Cx43 phosphorylations regulate its

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properties including assembly, trafficking, turnover, electrical and metabolic coupling.[1] Factors and conditions altering the phosphorylation pattern of Cx43 can also alter its properties and, by extension, affect heart function. Fibroblast growth factor-2 (FGF-2), a potent mitogenic and cytoprotective agent, is one such candidate factor. Its cardioprotective effects have been well documented.[7] When given to the normal heart, it acts as a pre-conditioning agent, and prevents ischaemia and reperfusion-associated cardiomyocyte damage and contractile dysfunction.[8,9] When given after the onset of ischaemia, FGF-2 acts as a post-conditioning agent by decreasing ischaemia and/or reperfusion-associated cell death and loss-of-function;[10–12] these processes are dependent on protein kinase C (PKC) activity. There is as yet no information as to whether the FGF-2 protection extends to preservation/restoration of Cx43 integrity during ischaemia and reperfusion.

Previously we showed that FGF-2 activates cardiomyocyte PKCϵ, increases physical interaction between Cx43 and PKCϵ, and upregulates relative levels of Cx43 phosphorylated at S262 and S368.[13–15] Here we show that additional protective treatments such as ischaemic PC, diazoxide administration, and post-ischaemic FGF-2 are also inducing Cx43 phosphorylation at S262 and S368, and that preventing phosphorylation of Cx43 at S262 increases vulnerability to ischaemic injury and prevents cytoprotection by PKCϵ or FGF-2. Our data indicate that P*Cx43 marks an injury-resistant cardiac state induced by PKC-activating treatment such as ischaemic PC or FGF-2, and suggest that phospho- (P) -S262-Cx43 acts as an effector of PKCϵ cardioprotection.

2. Methods

The investigation conforms with the Guide for the Care and Use of Laboratory Animals by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Approval was granted by the Protocol Management and Review Committee of the University of Manitoba. This section summarizes key methods. A full description can be found in the online Supplementary material.

2.1 Animals

Male Sprague–Dawley rats (200–250 g), and one-day-old rat pups were provided by the Central Animal Care Facility at the University of Manitoba.

2.2 Perfused isolated heart

Perfused isolated heart preparations and functional measurements were as described, using a Krebs–Henseleit (K–H) perfusion solution.[8,12]

2.3 Experimental design

Various treatments of isolated perfused hearts are described in *Figure 1*. All groups were subjected to 20 min perfusion with oxygenated K–H (stabilization) before treatment: ischaemic PC (3 cycles of 3 min global ischaemia, followed by 5 min reperfusion with K–H); diazoxide (150 μM); FGF-2 (10 μg/heart).

2.4 SDS—PAGE, western blotting, and immunofluorescence

As described previously.[15,16] Antibodies for total Cx43 (P.AB), dephosphorylated (D) -Cx43 (#13-8300), and phospho- (P) S262-Cx43, or P-S368-Cx43 have been validated.[3,5,14,15,17,18]

2.5 Cell death and injury

The Lactate Dehydrogenase (LDH) Assay and *in situ* cell death detection (TUNEL assay) kits were from Roche Diagnostics. Each coverslip ($n = 6$/group) was divided in eight fields, and total as well as TUNEL-positive myocyte nuclei were scored per field. TUNEL staining = fraction of TUNEL-positive cardiomyocytes per coverslip. LDH measurements were in colorimetric optical density units at 490 nM.

2.6 Adenoviral vectors

Adenoviral (Ad) vectors have been described.[13,18] Ad-S262A-Cx43 and Ad wild-type (WT) Cx43 were used at a multiplicity of infection (m.o.i.) of 2. Ad-PKCϵ was used at 20 m.o.i., as described previously.[19,20]

2.7 Simulated ischaemia

Cardiomyocytes isolated from one-day-old rat pups,[14] were plated at 800 000 cells/35 mm plate, in 10% bovine calf serum in Dulbecco's minimal essential medium (DMEM), and maintained at 37°C, in 5% CO_2 in room air (=non-ischaemic conditions). Next day, cells, forming a confluent, contractile layer, were infected with Ad vectors. One day later cultures were subjected to 'simulated ischaemia' by: transfer to pre-gassed (95% N_2, 5% CO_2) 'ischaemic medium' (in mM: 118 NaCl, 24 $NaHCO_3$, 1 NaH_2PO_4 H_2O, 2.5 $CaCl_2{\cdot}2H_2O$, 1.2 $MgCl_2$, 0.5 sodium EDTA $2H_2O$, 20 sodium lactate, and 16 KCl, pH 6.2)[21] plus incubation in a hypoxia chamber (<1% oxygen) for 6 h. FGF-2 (10 ng/ml) was added for 30 min before simulated ischaemia.

2.8 Statistical analysis

Differences between groups were compared using the Student's *t*-test (unpaired) and analysis of variance (ANOVA); $P < 0.05$ and $P < 0.01$ were considered significant and very significant, respectively (Graph-InStat). Two-way ANOVA (factors: simulated ischaemia and 'treatment'), and Duncan's method for pairwise multiple comparisons were used for data shown in *Figures 8B* and 9 (Sigma-Stat). Data are presented as means ± SD.

3. Results

We examined the effect of various cardioprotective manipulations (*Figure 1*) on cardiac Cx43 phosphorylation. Cx43 phosphorylation was assessed by western blotting and antibodies detecting: all Cx43 phosphorylation states, at 41–45 kDa (P.AB); only dephosphorylated D-Cx43, at 41 kDa (#13-8300); only P-S262-Cx43; only P-S368-Cx43. Cx43 migrating at 43–45 kDa, representing more extensively phosphorylated Cx43, is referred to as P-Cx43.

3.1 Effect of ischaemic PC and diazoxide

Lysates from normal perfused hearts analyzed under non-saturating protein loading conditions (20 μg/lane) detect a Cx43 band at 45 kDa (*Figure 2A*, P.AB). Under protein overload conditions P.AB also detect a band at 41 kDa (inset, *Figure 2A*). Ischaemic PC did not affect total Cx43 (*Figure 2A* and *A-i*). Normal hearts showed very faint or no staining for P-S262-Cx43, and some staining for P-S368-Cx43, as expected[15,22] (*Figure 2B* and *C*). Ischaemic PC hearts displayed significant increases in both P-S262-Cx43, and P-S368-Cx43 compared with controls (*Figure 2B* and *C*, and, respectively, *B-I* and *C-i*).

P-S368-Cx43 migrated predominantly near 41 kDa (*Figure 2C*), as reported.[22,23] P-S262-Cx43 migrated mainly at and/or just above 45 kDa (*Figure 2B*), indicating that it belongs to Cx43 population(s) phosphorylated at

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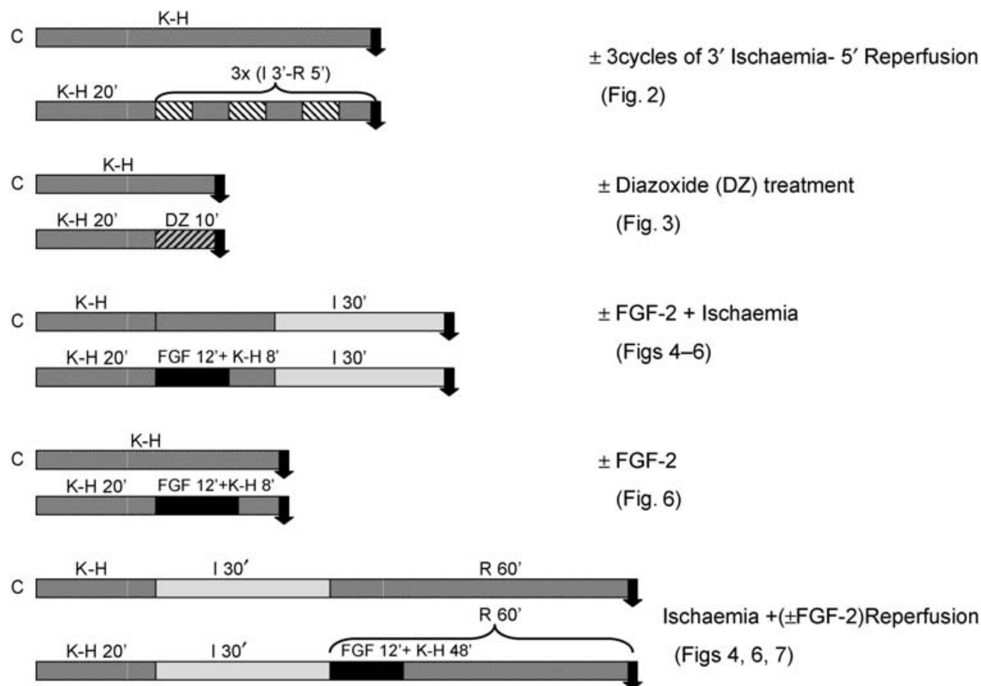

Figure 1 Experimental design. Five types of isolated perfused heart experiments, each with its own controls (C) were conducted to determine the effects of specific treatments on Cx43, as indicated. Duration of each treatment is indicated in minutes (′). K–H denotes perfusion with Krebs–Henseleit buffer. I, global ischaemia, R, reperfusion. Arrows mark termination of experiment, followed by Cx43 analysis.

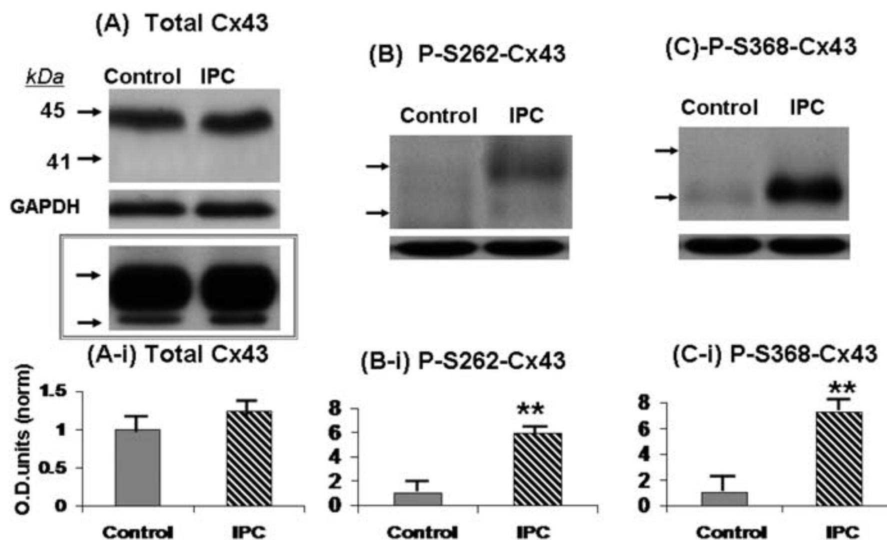

Figure 2 Ischaemic preconditioning promotes Cx43 phosphorylation at PKC target sites. (*A–C*) Representative western blots of cardiac lysates of control hearts and hearts subjected to ischaemic preconditioning (IPC), and probed, respectively, with antibodies recognizing total Cx43 (P.AB), P-S262-Cx43, P-S368 Cx43. Lysates were analyzed at 20 μg/lane, except in (A), inset, at 50 μg/lane. Staining for anti-glyceraldehyde phosphate dehydrogenase (GAPDH), indicative of even loading, is included. (A-i, B-i, C-i) Quantitative data, from densitometry measurements, corresponding to (A–C). Optical density, O.D. units, were normalized against control values, defined arbitrarily as 1. Migration of molecular weight markers is indicated in kDa. $^{**}P < 0.01$, $n = 6$.

several other residues, but not S368. Slower migration of P-S262-Cx43, corresponding to extensively phosphorylated Cx43 (P2) has been reported previously.[13,18,23] Differences in migration of P-S262-Cx43 and P-S368-Cx43 suggest that phosphorylation at these sites may be mutually exclusive, and that they belong to distinct Cx43 sub-populations.[15] We will be referring to above normal (non-stimulated) levels of P-S262-Cx43 and P-S368-Cx43 as P*Cx43.

Lysates from hearts perfused briefly with diazoxide, a treatment shown by others to be cardioprotective by a mechanism requiring PKCϵ[24] displayed elevated levels of both P-S262-Cx43, as well as P-S368-Cx43 compared with vehicle-perfused controls (*Figure 3A* and *B*).

In parallel studies we confirmed that ischaemic PC hearts show: (i) relatively improved recovery of contractile function after global ischaemia (30 min) and reperfusion (60 min) (Supplementary material online, *Figure S1*); (ii) protection

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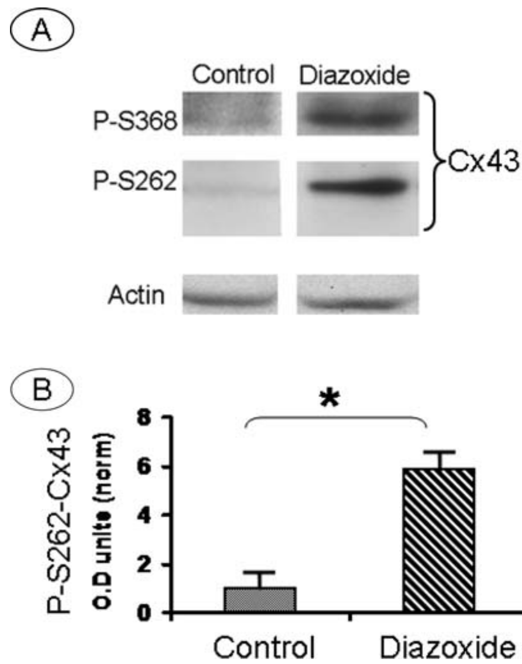

Figure 3 Diazoxide promotes Cx43 phosphorylation at S262, S368. (*A*) Representative western blots of cardiac lysates from control and diazoxide-treated hearts, probed for P-S262-Cx43, P-S368-Cx43, and pan-actin, as indicated. P-S368-Cx43 images are representative of $n = 2$. (*B*) Corresponding quantitative data for P-S262-Cx43 (O.D. units) normalized against control values. $^{*}P < 0.01$, $n = 4$.

from ischaemia-induced Cx43 dephosphorylation and lateralization (Supplementary material online, *Figure S2*).

3.2 Effect of ischaemia (±FGF-2), and ischaemia-reperfusion (±FGF-2) on relative levels of P-Cx43 and D-Cx43

Our results are shown as representative western blots (*Figure 4A–C*) and corresponding quantitative data (*Figure 4A1* and *2*, *B1* and *2*, *C1* and *2*). Probing with P.AB showed that relative levels of total Cx43 were similar between non-ischaemic controls and hearts subjected to ischaemia (± FGF-2 pre-treatment), *Figure 4A*, *B*, *A1*, or hearts subjected to ischaemia and reperfusion (± FGF-2), *Figure 4A*, *C*, *A2*. Taken together, our data indicated that there was no net Cx43 loss (degradation) during our experiments.

Probing with P.AB showed that pre-ischaemic hearts are composed of P-Cx43 (*Figure 4A*). Ischaemia resulted in disappearance of P-Cx43 and accumulation of 41–42 kDa Cx43 (*Figure 4B*). This was partially prevented by FGF-2 pre-treatment, resulting in 78% P-Cx43 despite global ischaemia (*Figure 4B* and *B1*). Hearts subjected to ischaemia-reperfusion accumulated mainly 41 kDa Cx43 after 60 min of reperfusion, with minimal recovery (15% of total) of P-Cx43 (*Figure 4C* and *C1*). In contrast, FGF-2 reperfused hearts accumulated 80% P-Cx43 (*Figure 4C* and *C1*). FGF-2-reperfusion of ischaemic hearts therefore reversed the effects of ischaemia and re-established prominence of P-Cx43.

Confirming results with P.AB, the #13-8300 antibodies detected a prominent 41 kDa band, D-Cx43, in ischaemic hearts (*Figure 4B*); and hearts subjected to ischaemia-reperfusion (*Figure 4C*). D-Cx43 was significantly decreased in the FGF-2-pre-treated ischaemic group (*Figure 4B* and *B2*); and the FGF-2-reperfused group (*Figure 4C* and *C2*), compared with their non-FGF-2-treated counterparts.

We used immunofluorescence staining to examine the effect of FGF-2 pre-treatment on total and D-Cx43 localization after ischaemia. Representative images are shown in *Figure 5*. As expected, non-ischaemic hearts showed Cx43 (P.AB) immunostaining at intercalated disks (arrows, *Figure 5A*), and lack of staining for D-Cx43 (*Figure 5A*–i). Ischaemia caused Cx43 redistribution to lateral cardiomyocyte surfaces (*Figure 5B*, double arrows), as well as strong immunoreactivity for #13-8300 (D-Cx43, *Figure 5B*–i). Morphological examination of several tissue sections, from several ischaemic hearts, indicated that most myocytes stained positive for #13-8300, and displayed some Cx43 lateralization. These changes were not observed in the FGF-2 pre-treated hearts (*Figure 5*, c/c-i).

3.3 Effect of ischaemia (±FGF-2), and ischaemia-reperfusion (±FGF-2) on relative levels of P*Cx43

As we showed before,[15] and repeat here for comparison, FGF-2 pre-treatment increased P-S262- and P-S368-Cx43 above baseline levels, thus inducing a P*Cx43 state before ischaemia (*Figure 6A1* and *B1*). After ischaemia, FGF-2 pre-treated hearts had significantly higher levels of P-S262-Cx43 or P-S368 Cx43 compared with non-treated hearts, as seen in *Figure 6A*2 and *3* or *Figure 6B*2 and *3*, respectively. Within the FGF-2-treated groups (*Figure 6C*), total levels of P-S262- or P-S368-Cx43 were not significantly affected by ischaemia, as seen in *Figure 6C1* and *2* and *Figure 6C3* and *4*, respectively.

A P*Cx43 state was also induced after ischaemia, during reperfusion with FGF-2 (*Figure 7A*). Significant up-regulation of anti-P-262-Cx43 (*Figure 7A* and *A1*) and anti-P-368-Cx43 (*Figure 7A* and *A2*) immunoreactive bands were observed in the FGF-2-reperfused compared with the K–H reperfused group. Reperfusion-associated P-S262-Cx43 migrated mostly at ~45 kDa, but diffuse immunoreactivity was also seen at ~42 kDa (*Figure 7A*). Reperfusion-associated P-S368-Cx43 migrated mostly at 41–42 kDa, but some anti-P-S368-Cx43 bands were also detected at ~45 kDa (*Figure 7A*). Our data indicate that phosphorylation patterns in Cx43 subpopulations after reperfusion are different to those of non-reperfused hearts; and that Cx43 phosphorylations at S262 and S368 may not be mutually exclusive in reperfusion-associated Cx43.

As expected,[11,12] treating hearts with FGF-2 during reperfusion improved contractile functional recovery; e.g. systolic pressure was increased by 35% compared with K–H-only reperfused hearts, measured at 60 min of reperfusion. Functional measurements from hearts used to analyze Cx43 (*Figures 4* and *7*) are described in another manuscript[25] and therefore are not included here.

3.4 Role of Cx43 and the S262 site in resistance to injury

To determine if the ability of Cx43 to become phosphorylated at S262 could affect cytoprotection, S262A-Cx43 (simulating constitutive lack of phosphorylation at S262),

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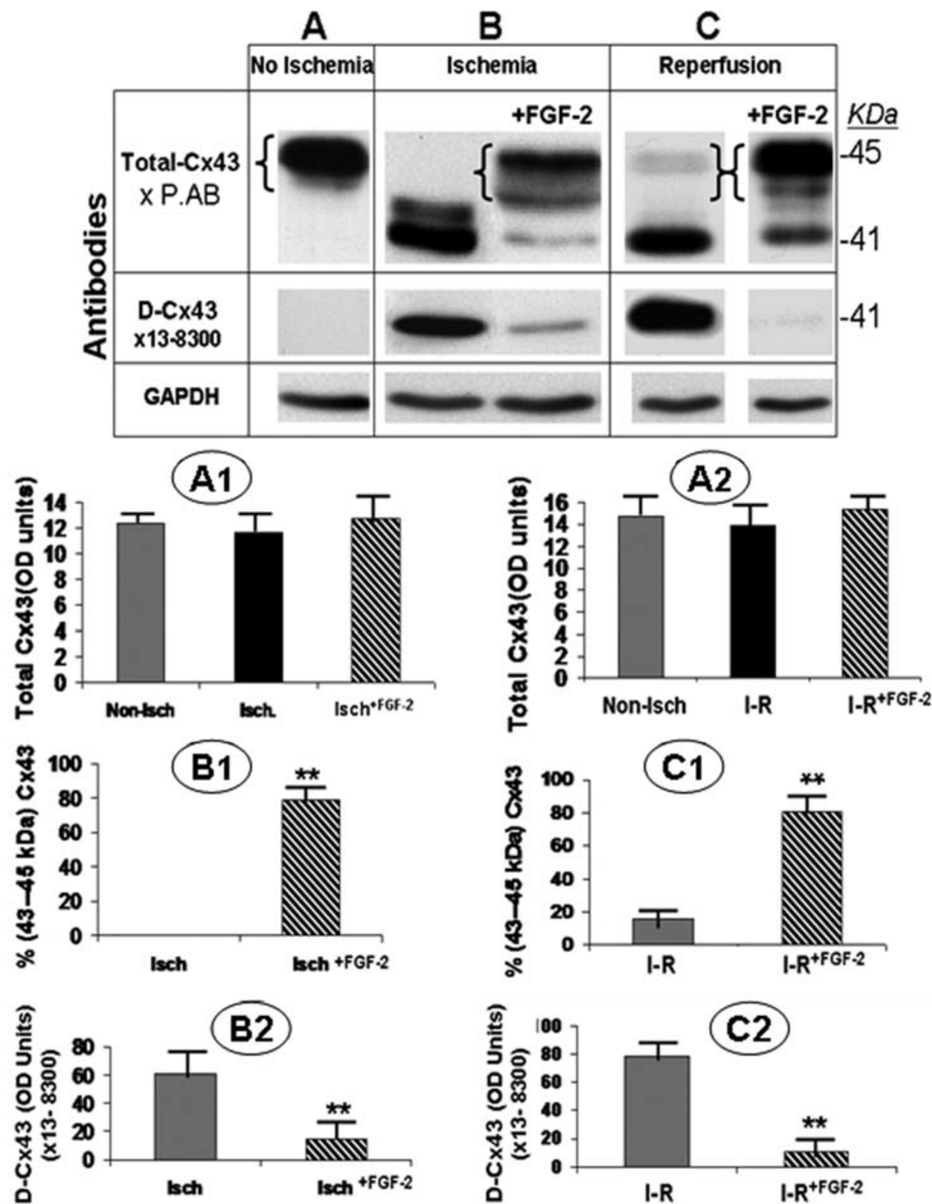

Figure 4 Effects of pre- or post-ischaemic FGF-2 on 41–45 kDa Cx43. Representative western blots from, (*A*), non-ischaemic hearts, (*B*), ischaemic hearts ± -FGF-2 pre-treatment, (*C*), hearts subjected to ischaemia (± FGF-2)-reperfusion. Blots were probed with antibodies for total Cx43 (P.AB), D-Cx43 (#13-8300), and GAPDH, as indicated. Brackets denote 43–45 kDa P-Cx43. (*A1* and *A2*): relative levels of total Cx43 (O.D. units, P.AB) in non-ischaemic hearts (non-Isch), and, (A1) ischaemic hearts ± FGF-2 pre-treatment (Isch, $Isch^{FGF-2}$), (A2), hearts subjected to ischaemia (± FGF-2)-reperfusion (I-R, $I\text{-}R^{FGF-2}$). (*B1*) and (*C1*) Percent 43–45 kDa P-Cx43 (P.AB) in, respectively, ischaemic hearts ± FGF-2 pre-treatment and hearts subjected to ischaemia, and reperfusion ± FGF-2. (*B2*) and (*C2*) Relative levels of D-Cx43 (#13-13800) in respectively, ischaemic hearts ± FGF-2 pre-treatment, and hearts subjected to ischaemia and (± FGF-2)-reperfusion; $n = 6$; $^{**}P < 0.01$.

or WT-Cx43 were introduced in confluent cardiomyocyte cultures by Ad gene transfer. After simulated ischaemia, Ad-S262A-Cx43 cultures showed significantly increased TUNEL staining and LDH release compared with Ad-vector-infected controls, as indicated (*Figure 8A1* and *2*). In contrast, Ad-WT-Cx43 cultures displayed reduced TUNEL staining and LDH release compared with Ad-vector controls (*Figure 8A1* and *2*).

We next asked whether preventing Cx43 phosphorylation at S262 would affect cytoprotection induced by overexpressed PKCϵ,[25] or by FGF-2 treatment. As seen in *Figure 8B1* and *2*, Ad-vector cultures subjected to simulated ischaemia had significantly increased TUNEL staining and LDH release compared with Ad-vector cultures kept in non-ischaemic conditions. PKCϵ overexpression prevented or reduced the effect of simulated ischaemia on, respectively, TUNEL staining and LDH release (*Figure 8B1* and *2*). Expression of S262A-Cx43 had the opposite effect, potentiating the deleterious effects of simulated ischaemia (*Figure 8B1* and *2*), as already seen in *Figure 8A1* and *2*. PKCϵ cytoprotection was abolished in the presence of S262A-Cx43; TUNEL staining or LDH release were as high in cultures co-expressing PKCϵ/S262A-Cx43 as those expressing only S262A-Cx43 (*Figure 8B1* and *2*). Similar findings were obtained with FGF-2

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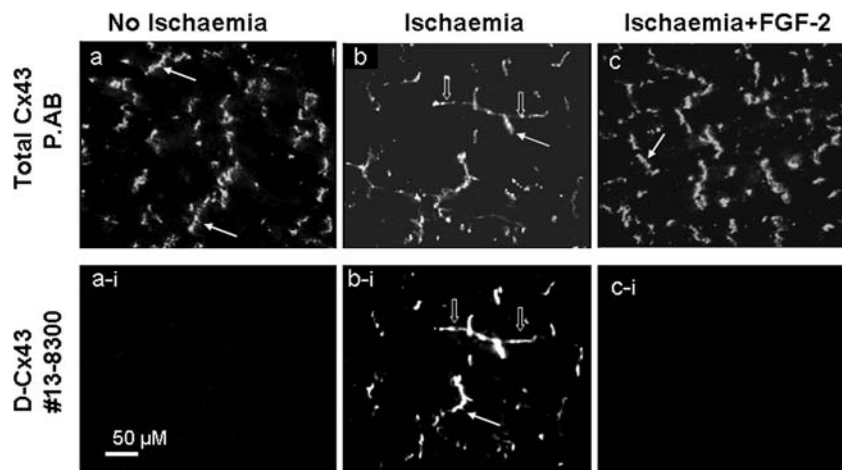

Figure 5 FGF-2 pre-treatment prevents ischaemia-induced Cx43 changes. Representative double-immunofluorescence images of sections from (*a*, *a–i*), control, non-ischaemic hearts, (*b*, *b–i*), hearts subjected to 30 min global ischaemia, (*c*, *c–i*) FGF-2-pre-treated hearts subjected to 30 min global ischaemia. Sections were probed for, panels (a–c), total Cx43 (P.AB), and, (a-i, b-i, c-i), D-Cx43 (#13-8300). Solid arrows point to intercalated disks, while 'empty' arrows point to lateral myocyte surfaces.

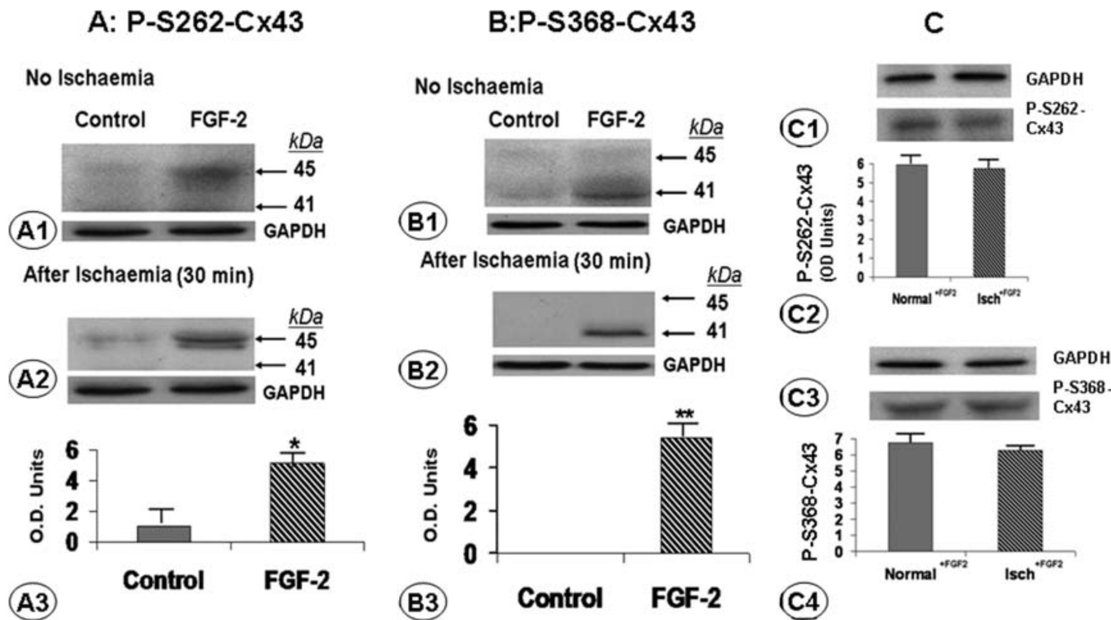

Figure 6 The effect of ischaemia, ± FGF-2 pre-treatment, on P*Cx43. (*A1* and *B1*, 'no ischaemia') panels show representative western blots from lysates of hearts perfused with oxygenated K–H (± FGF-2), probed for P-S262- and P-S368-Cx43, respectively. Corresponding GAPDH bands are included. (*A2* and *B2*) ('after ischaemia'): representative western blots from ischaemic hearts ± FGF-2 pre-treatment probed, respectively, for P-S262- and P-S368-Cx43. (*A3* and *B3*) show quantitation of relative P-S262- and P-S368-Cx43, respectively, corresponding to (A2) and (B2), in O.D. units. (*C1* and *C3*): representative western blots and corresponding quantitative data (O.D. units) for P-S262-Cx43 in FGF-2 pre-treated hearts before ischaemia (normal^{+FGF-2}), and after ischaemia (Isch^{+FGF-2}). (*C3* and *C4*): representative western blots and corresponding quantitative data for P-S368-Cx43 in FGF-2 pre-treated hearts before and after ischaemia. In all cases $n = 6$, $^{*}P < 0.05$, $^{**}P < 0.01$.

cytoprotection. As seen in *Figure 9*, FGF-2 pre-treatment significantly decreased TUNEL staining in Ad-Vector cultures subjected to simulated ischaemia, but was unable to reduce the effects of S262A-Cx43 expression (*Figure 9*).

Before simulated ischaemia, Ad-S262A-Cx43 or Ad-WT-Cx43-infected cultures displayed minimal levels of cell death or injury, similar to Ad-vector cultures (Supplementary material online, *Figure S3*). Infection with Ad-S262A-Cx43 and Ad-Wt-Cx43 resulted in modest overexpression, detected as a ~2.5-fold increase of total (endogenous plus introduced) immunoreactive Cx43 (Supplementary material online, *Figure S4*). Representative TUNEL stained cell images are included in Supplementary material online, *Figure S5*.

4. Discussion

The purpose of our studies was to investigate the relationship between PKC-dependent cardioprotection (ischaemic PC, pre-ischaemic or post-ischaemic FGF-2 treatment) and Cx43 phosphorylation at PKC sites such as S262. We also

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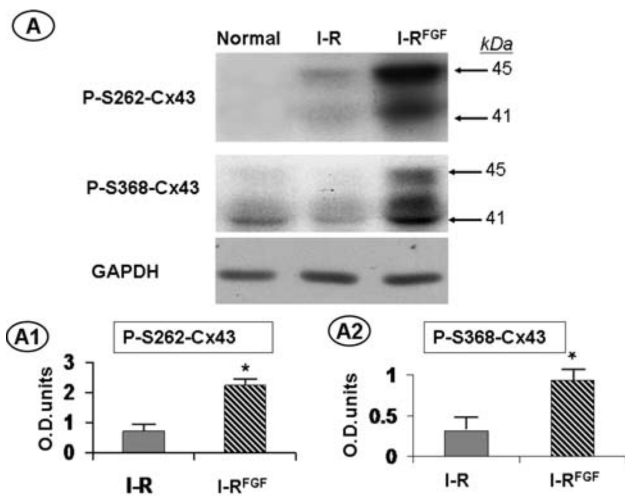

Figure 7 P*Cx43 is induced by FGF-2 during reperfusion. (*A*) Representative western blots from non-ischaemic hearts (normal), and hearts subjected to ischaemia and (±FGF-2) reperfusion (I-R, I-R^{FGF}); probed for P-S262-, P-S368-Cx43, and GAPDH, as indicated. (*A1* and *A2*) show corresponding quantitative data (O.D. units) for relative P-S262- or P-S368-Cx43; $n = 6$, $*P < 0.05$.

examined a possible cause-and-effect relationship between Cx43 phosphorylation at S262 and resistance to injury.

4.1 P*Cx43 is a common feature in models of PKC-dependent acute cardioprotection

Several studies indicated an essential role for Cx43 in developing ischaemic PC-type cardioprotection.[22,26–29] There is, however, limited information about Cx43 molecular modifications associated with cardioprotection. We have now shown that several experimental treatments promoting PKC-dependent cardioprotection are also causing above normal Cx43 phosphorylation at PKC target sites. Ischaemic PC, FGF-2 treatment before ischaemia[15] as well as FGF-2 treatment post-ischaemia were characterized by robust increases in P-S262- and P-S368-Cx43, compared to normal hearts. The same was true when hearts were perfused with diazoxide, a compound reported to promote ischaemic PC-like cardioprotection.[24]

Phosphorylations at the S262- and S368- sites of Cx43 are both dependent on a central mediator of cardioprotection, PKCϵ[13,14,18,22,30] and may thus serve as 'markers' for development of an injury-resistant state. Both sites regulate Cx43 properties: phosphorylation at S368 decreases coupling[31,32] while S262 regulates Cx43-signalling functions that do not necessarily depend on GJ, such as inhibition of DNA synthesis.[13,18] Both S262 and S368 sites have been previously identified as being capable of phosphorylation within the cell.[13,33] Their relative levels, however, are comparatively low in the non-'protected' myocardium[15,22] indicating that the normal, in contrast to the 'injury-resistant' state, does not support extensive constitutive Cx43 phosphorylation at these residues. To our knowledge, this is the first time that ischaemic PC or diazoxide are shown to promote acute and significant increases in P-S262- and P-S368-Cx43 in the normal heart. It is also the first time that these phosphorylations are shown to be inducible during reperfusion by FGF-2. Nevertheless, our data are in broad agreement with a report that a delta-opioid receptor agonist, which promotes PKCϵ-mediated cardioprotection, increased cardiac P-S368-Cx43 examined only during early ischaemia.[22]

Loss of ATP during ischaemia and subsequent activation of phosphatases result in Cx43 dephosphorylation.[16] The preservation of the P*Cx43 state even after 30 min ischaemia implies either that phosphatase activation did not occur or that P*Cx43 became resistant/inaccessible to phosphatases. Both possibilities merit consideration. Global cytoprotective pathways activated by FGF-2 would preserve mitochondrial integrity and energy stores and prevent activation of phosphatases. It is also known that FGF-2 renders Cx43 inaccessible to antibodies recognizing epitopes within residues 260–270, a region containing the S262 site.[34] Phosphorylation of Cx43 at S368 causes conformational changes decreasing accessibility to trypsin.[35] It is logical to expect that molecular changes causing 'masking' of Cx43 domains from antibodies or proteolytic enzymes may also protect those domains from phosphatases.

The ability of P*Cx43 to remain elevated even after 30 min of ischaemia shows that an inducible molecular signal is maintainable for some time and relays the effects of the pre-ischaemic stimulus into the reperfusion stage. Such relayed effects likely include reduced metabolic coupling, proposed to mediate the protective and anti-arrhythmogenic effect of ischaemic PC.[27] A similar suggestion was made about the role of increased phosphorylation of Cx43 at S368 in response to an opioid receptor agonist.[22] In contrast to these reports implicating GJ in development of cardioprotection,[22,27] Li and colleagues concluded that GJ may not be essential for ischaemic PC,[36] since isolated cardiomyocytes can be preconditioned. Nevertheless, as has been pointed out,[22] the magnitude of protection in isolated myocytes is not as robust as in whole hearts, suggesting that isolated cells may be regulated differently. We think it likely that Cx43 can contribute to ischaemic PC-type protection by both GJ-dependent and independent mechanisms. There is indeed strong evidence that Cx43 hemi-channels, and mitochondrial Cx43 confer GJ-independent cytoprotection.[37]

GJ uncoupling is considered beneficial during reperfusion because it prevents the spread of injurious stimuli culminating in calcium overload and cell death.[26,38] It is reasonable to suggest that post-conditioning cardioprotection by FGF-2[10–12] may be mediated, at least in part, by its ability to induce P*Cx43 during reperfusion.

In addition to promoting P*Cx43, FGF-2 attenuated the ischaemia-induced accumulation of D-Cx43; post-ischaemic FGF-2 also significantly reduced D-Cx43. Reducing relative levels of D-Cx43 would be expected to preserve the structural integrity of intercalated disks.[3] It would also prevent undesirable increases in permeability not only of GJ but also hemi-channels. While hemi-channels are normally closed, Cx43 dephosphorylation causes them to open, resulting in injury and death during cardiomyocyte ischaemia-reperfusion.[39]

4.2 The ability of Cx43 to be phosphorylated at S262 contributes to the ability of cardiomyocytes to develop resistance to injury

Having shown that increased P-S262-Cx43 is associated with several PKC-dependent cardioprotective treatments, we used cultured cardiomyocytes to test whether the ability of

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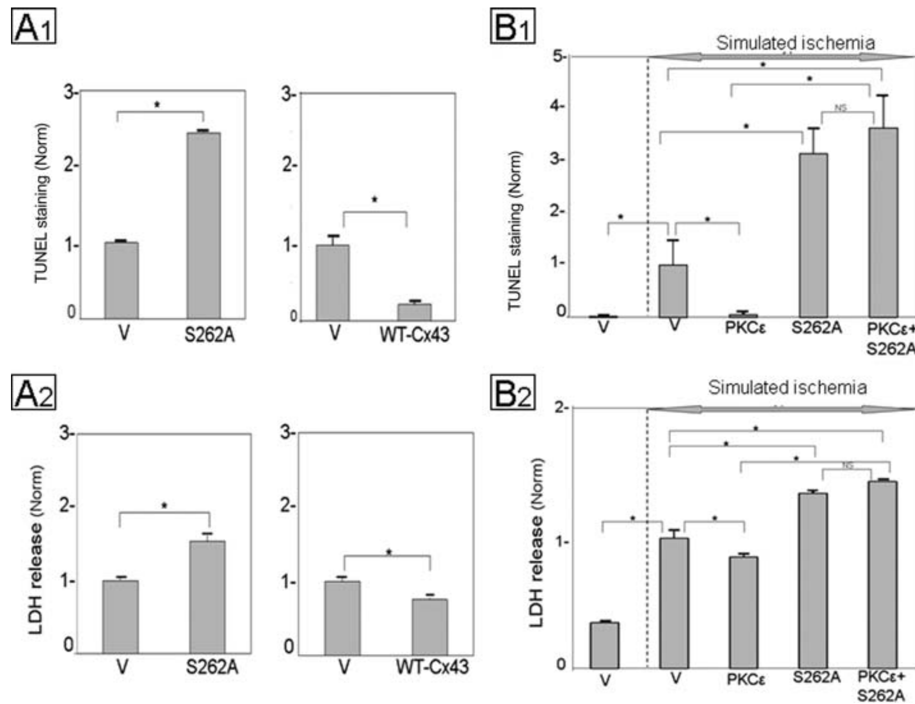

Figure 8 The effect of S262A-Cx43 on, (*A1* and *A2*) simulated ischaemia-induced cardiomyocyte cell death and injury, and, (*B1* and *B2*), PKCϵ-associated cytoprotection. (A1 and A2) Fold-change in cell death (TUNEL staining, Norm), or injury (LDH release, Norm) after simulated ischaemia in a set of cultures infected with Ad-Vector (V) or Ad-S262-Cx43 (S262); and a parallel set of cultures infected with Ad-Vector(V) or wild-type Cx43 (WT-Cx43). $^*P < 0.05$, $n = 6$. (B1 and B2) Fold-change in TUNEL staining (Norm), and LDH release (Norm) in: cultures infected, as indicated, with Ad-Vector (V), Ad-PKCϵ (PKCϵ), Ad-S262-Cx43 (S262), or Ad-PKCϵ and Ad-S262A-Cx43 (PKCϵ+S262A), and subjected to simulated ischaemia. Double-pointed arrows point to values from groups subjected to simulated ischaemia. Brackets indicate comparisons between groups ($n = 6$, $^*P < 0.05$). Data were normalized giving a value of one to measurements from Ad-vector cultures subjected to simulated ischaemia. The fraction of TUNEL-positive nuclei after simulated ischaemia in Ad-Vector cultures was at 20–25%; corresponding arbitrary LDH O.D. units (490_{nm}) varied between 0.9 and 1.2.

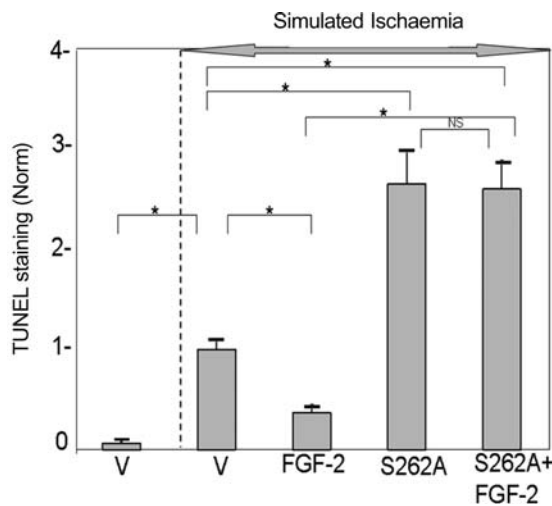

Figure 9 S262A-Cx43 expression prevents FGF-2 cytoprotection: Fold-change in cell death [TUNEL staining (norm)] in cultures infected with Ad-vector (V) or Ad-S262A-Cx43 (S262A), ±FGF-2 pre-treatment, and subjected to simulated ischaemia (double-pointed arrows), as indicated. $^*P < 0.05$, $n = 6$. Ad-vector cultures subjected to simulated ischaemia displayed 23% TUNEL-positive nuclei.

Cx43 to become phosphorylated at S262, an event that occurs downstream of PKCε activation, is mediating cytoprotection. To decrease the proportion of Cx43 that can become phosphorylated at S262, we expressed S262A-Cx43, a mutated molecule incapable of phosphorylation at that site. Since expression of S262A-Cx43 but not similar levels of expression of WT-Cx43 increased cardiomyocyte vulnerability to ischaemic-type injury, we conclude that the majority of cellular Cx43 needs to be capable of phosphorylation at S262 in order for 'baseline' levels of injury-resistance to be maintained. Similarly, as neither FGF-2 nor increased PKCϵ were capable of inducing cytoprotection in the presence of S262A-Cx43 expression, we propose that their protective effects depend on being able to promote phosphorylation of the majority of cellular Cx43 at S262, at least in the cardiomyocyte culture model used here.

Our data imply that even modest overexpression of the S262A-Cx43 has a 'dominant-negative' effect over endogenous Cx43. S262A-Cx43, having an intact channel-forming domain, can interact with endogenous Cx43 to form mixed, and thus 'altered', connexons or aggregates. Studies have shown that all six components of a connexon need to be capable of phosphorylation to achieve full effect on connexon properties.[32] It is possible that all Cx43 monomers in an aggregate may need to become phosphorylated at S262 for developing PKCϵ-mediated cytoprotection.

The effect of S262A-Cx43 is not due to overexpression because similar levels of expression of WT-Cx43 elicited protection against injury, in agreement with studies showing a positive relationship between Cx43 levels and cardioprotection.[28,37] Furthermore, S262A-Cx43 localizes in a manner similar to its wild-type counterpart, shown for both Cx43-deficient,[18] and Cx43 expressing cells such as myocytes[13] (as also seen in Supplementary material online, *Figure S3*), thus its effects are not likely to result from aberrant localization.

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Phosphatase inhibition exerts cardioprotection in the absence of a preconditioning stimulus.[40] It would be pertinent to examine whether phosphatase inhibitors increase baseline levels of P-S262-Cx43, an event which, based on our data, would promote cytoprotection.

The precise mechanism by which Cx43 phosphorylation at S262 contributes to cardioprotection needs to be determined. The effect may be a consequence of subtle changes in GJ coupling, and/or Cx43 protein–protein interactions. Alternatively, or concurrently, it is possible that Cx43 hemi-channels and/or mitochondrial Cx43 are involved. Certainly, PKCϵ is present at all subcellular sites where Cx43 is found including, intercalated disks, plasma membrane, and mitochondria, and thus it is probable that it can stimulate above normal Cx43 phosphorylation at S262 at all these locations. We suggest that because Cx43 is capable of influencing cell behaviour at multiple levels it is likely to be an important effector of PKCϵ cardioprotection.

Supplementary material

Supplementary Material is available at *Cardiovascular Research* online.

Conflict of interest: none declared.

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